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Morphological and genetic characterisation of *Beauveria sinensis* sp. nov. from China

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Abstract —*Beauveria sinensis* sp. nov. was isolated from a larva of *Geometridae* (*Lepidoptera*) collected from Tiantangzhai, Anhui province, China. It is characterized by elongated ellipsoidal to cylindrical conidia, solitary conidiogenous cells that are cylindrical or with slightly swollen bases, white to pale pink colony in culture, and small mycelial pellets consisting of curved hyphae. Phylogenetic analyses of translation elongation factor-1 alpha (TEF), RNA polymerase II largest subunit (RPB1), and RNA polymerase II second largest subunit (RPB2) sequence data support it as a unique species and sister to *B. sungii*. It does not, however, form a monophyletic group with other elongated ellipsoidal to cylindrical conidia-producing species in *Beauveria*. Type isolates and holotype are deposited in the Research Center for Entomogenous Fungi of Anhui Agricultural University (RCEF).

Key words —*Ascomycetes*, taxonomy, entomopathogenic fungi

Introduction

Beauveria Vuill. is a globally distributed genus of soil-borne and entomopathogenic hyphomycetes. It is of particular interest as a model system for the study of entomopathogenesis and the biocontrol of pest insects (Rehner & Buckley 2005). *Beauveria* species are characterized by whorled conidiophores and dense clusters of sympodial and globose or flask-shaped short conidiogenous cells with apical denticulate rachi that give rise to one-celled hyaline conidia. Conidial shape in *Beauveria* can be globose, ellipsoidal, cylindrical, or comma-shaped.

Recent phylogenetic analyses of *Beauveria* inferred from partial sequences of RNA polymerase II largest subunit (RPB1), RNA polymerase II second largest subunit (RPB2), translation elongation factor-1 alpha (TEF) genes, and the Bloc nuclear intergenic region (Bloc) resolved 12 clades, each of which

includes one recognized species (Rehner et al. 2011). Since then Zhang et al. (2013) have described *B. lii* Sheng L. Zhang & B. Huang from China, a species with large cylindrical to ellipsoidal conidia. In *Beauveria*, there are eight species with ellipsoidal, cylindrical, or comma-shaped conidia: *B. amorpha* (Höhn.) Minnis et al., *B. asiatica* S.A. Rehner & Humber, *B. brongniartii* (Sacc.) Petch, *B. caledonica* Bissett & Widden, *B. lii*, *B. malawiensis* S.A. Rehner & Aquino de Muro, *B. sungii* S.A. Rehner & Humber, and *B. vermiconia* de Hoog & V. Rao (Bissett & Widden 1988, de Hoog & Rao 1975, Rehner et al. 2006, 2011; Samson & Evans 1982, Shimazu & Hashimota 1988, Zhang et al. (2013). Five species with globose to subglobose conidia include the type of the genus, *B. bassiana* (Bals.-Criv.) Vuill., and *B. australis* S.A. Rehner & Humber, *B. kipukae* S.A. Rehner & Humber, *B. pseudobassiana* S.A. Rehner & Humber, and *B. varroae* S.A. Rehner & Humber (Rehner et al. 2011).

Recently, we isolated a *Beauveria* with elongated ellipsoidal to cylindrical conidia from Anhui province, China, infecting *Geometridae* larvae. The isolate possessed unique cultural and morphological characteristics and did not form a monophyletic group with other *Beauveria* species that produce cylindrical conidia. We concluded that it represents a new species and describe it here as *B. sinensis*.

Materials & methods

Collection of specimens and isolation

An entomogenous specimen (TTZ070716-26) was collected from Tiantang zhai, Jinzhai county, Anhui province, China, in July 2007. The host was identified as belonging to *Geometridae* (*Lepidoptera*). Strain RCEF3903 was isolated from this collected specimen on SDAY (1% w/v peptone, 4% w/v dextrose, 0.2% w/v yeast, 1.5% w/v agar) medium.

Strain identification

Morphological characteristics are based on cultures incubated on quarter-strength SDAY (SDAY/4) at 25 °C for 14 d. The isolated fungus was examined using classical mycological techniques based on growth rate as well as macroscopic and microscopic characteristics. The ex-type culture and a dried-culture holotype specimen has been deposited in the Research Center for Entomogenous Fungi, Anhui Agriculture University, Hefei, Anhui, China (RCEF).

DNA extraction, PCR and sequencing

Conidia were inoculated onto SDAY medium overlaid with a disc of sterilized cellophane. After incubating at 25 °C for approximately 7 days, genomic DNA was extracted from the mycelia scraped from the cellophane using modified CTAB method (Gardes & Bruns 1993, Spatafora et al. 1998). The extracted DNA was stored in sterile distilled water to a final concentration of 1–2 ng μl^{-1} and stored at –20 °C.

To perform phylogenetic analysis of the isolated *Beauveria* strains (Zhang et al. 2013; Rehner et al. 2011), the partial sequences of three nuclear loci, including TEF, RPB1,

and RPB2, were amplified as described by Rehner & Buckley (2005) and Rehner et al. (2011). After purifying PCR product using EasyPure quick gel extraction kit (TransGen Biotech), DNA sequencing was performed at Sangon Company (Shanghai, China) and the resulting sequences of RCEF3903 were submitted to GenBank.

Sequence alignment and phylogenetic analyses

DNA sequences generated in this study were assembled and edited using Codoncode Aligner (version 3.6.1). Sequences of TEF, RPB1, and RPB2 from 69 taxa (68 *Beauveria* isolates and one *Isaria tenuipes* strain as outgroup) based on the results of Zhang et al. (2013), Rehner & Buckley (2005), and Rehner et al. (2006) were downloaded form GenBank. Multiple sequence alignments for TEF, RPB1, and RPB2 constructed using Clustal X 1.81 (Thompson et al. 1997) were concatenated into a single file using the program Mafft (Katoh et al. 2002), and MEGA was used to output a Nexus file for phylogenetic analysis. The datasets were analysed using both maximum parsimony (MP) and Bayesian algorithms.

PAUP* 4.0b10 (Swofford 2002) was used to perform maximum parsimony analyses on the combined dataset of TEF, RPB1, and RPB2 with 1000 replicates of heuristic search of random sequence additions, branch swapping algorithm by tree bisection-reconnection (TBR) and MulTrees on. Insertions and deletions were minimized by direct examination and treated as missing data. Ambiguously aligned sequence regions were excluded from the data matrix before analysis. Branch support was estimated by bootstrapping using 1000 replicates with full heuristic search (Felsenstein 1985). Clades with bootstrap values ≥70% were considered strongly supported by the data.

Bayesian analyses were conducted with an online version of MrBayes 3.0b4 (Huelsenbeck et al. 2001) through the CIPRIS web portal. 1,000,000 Markov Chain Monte Carlo (MCMC) generations were performed with four chains (three cold, one heated) and trees were saved every 100 generations for a total of 100,000 trees. Analyses were repeated two times and converged close to the same value (the standard deviation of split frequencies about 0.005) and first 25% of trees were discarded as burn-in. The consensus tree with the support values for each branch constituting their posterior probability (≥95% were considered as significantly supported by the data) was combined into a single tree file. Posterior probabilities for branches receiving ≥95% support are reported below the respective branches on the single best tree from the Bayesian analysis (Fig. 1). The bootstrap values for branches supported in ≥70% are listed above branches in Fig. 1.

Results

For RCEF3903, PCR amplification yielded TEF, RPB1, and RPB2 amplicons of 1714 bp, 2866 bp, and 2118 bp. The combined alignment of the above genes included a total of 6698 characters. After ambiguously aligned positions excluded, the final alignment comprised 6028 bp (TEF: 1004; RPB1: 2860; RPB2: 2164), of which 771 were parsimony informative. Maximum parsimony analysis of 70 taxa dataset yielded 48 equally most-parsimonious trees of 2018 tree length with consistency indices (CI) of 0.7096 and retention indices (RI) of 0.9091. One of nine equally most-parsimonious trees is shown in Fig. 1 with

bootstrap values ≥70% above the relevant branches. Bayesian analysis resulted in the same topology as the MP analysis shown in Fig. 1.

The phylogenetic analysis of combined data set well supported RCEF3903 as a sister group to the *B. sungii* clade containing seven individuals. All isolates from this clade produced ellipsoidal or oblong conidia in culture (Rehner et al. 2011), and RCEF3903 differs from these seven isolates in conidial size. The molecular data and analyses also suggest a wide relationship of RCEF3903 with *B. caledonica* group, *B. amorpha* group, *B. malawiensis* group and *B. lii*.

Taxonomy

Beauveria sinensis Ming J. Chen, Z.Z. Li & B. Huang, **sp. nov.** Plates 2–7

MycoBank MB 801325

Differs from all other *Beauveria* species by its pale pink colony and production of mycelial pellets consisting of curved hyphae.

Type: China, Anhui Province, Jinzhai County, isolated from a larva of *Geometridae*, July 2007, coll. Mingjun Chen (**Holotype**, RCEF3903-DAC1; ex-type culture RCEF3903; GenBank HQ270151, JX524283, JX524284).

Etymology: *sinensis,* for the country of origin, China.

Colony on SDAY/4 attaining a diameter of 23–31 mm after 14 days at 25 °C, aerial mycelium white, dense and cottony at first, slowly becoming pale pink and producing considerable amounts of mycelial pellets on older portions, regular in the margin, reverse light yellow-brown, odor indistinct. The mycelial pellets attaining 95.0–150.0 µm in diameter after 14 days at 25 °C, and consisting of curved hyphae. Typical hyphae hyaline, smooth-walled, septate, branched, 1.8–2.5 µm diam., curved mycelium a little slighter. Conidiophores arising on aerial hyphae, similar to aerial hyphae, mostly simple. Conidiogenous cells mostly borne on conidiophores, occasionally on aerial mycelium, solitary, flask-shaped to cylindrical, 8.5–20.0 × 1.6–3.0 µm, tapering into a long slender denticulate rachis, geniculate or irregularly bent, 5.0–10.0 × 0.5–0.6 µm. Conidia elongate ellipsoidal to cylindrical, one-celled, hyaline, smooth, 3.0–5.0 × 1.5–2.0 µm (L^m = 4.1 µm, W^m = 1.7 µm, Q^m = 2.4), chlamydospores absent. Teleomorph not observed.

Discussion

We tried to amplify the Bloc loci of *B. sinensis* several times with Bloc primers described by Rehner et al. (2011), but failed to get its PCR production. The phylogenetic relationship among the *Beauveria* inferred from three nuclear loci was mainly consistent with those inferred from four nuclear loci (Rehner et al. 2011). Furthermore, Rehner et al. (2011) emphasized that each of the four loci used to reconstruct the phylogeny of *Beauveria* was effective for accurate diagnosis of all 12 species based on multiple species-specific phylogenetically

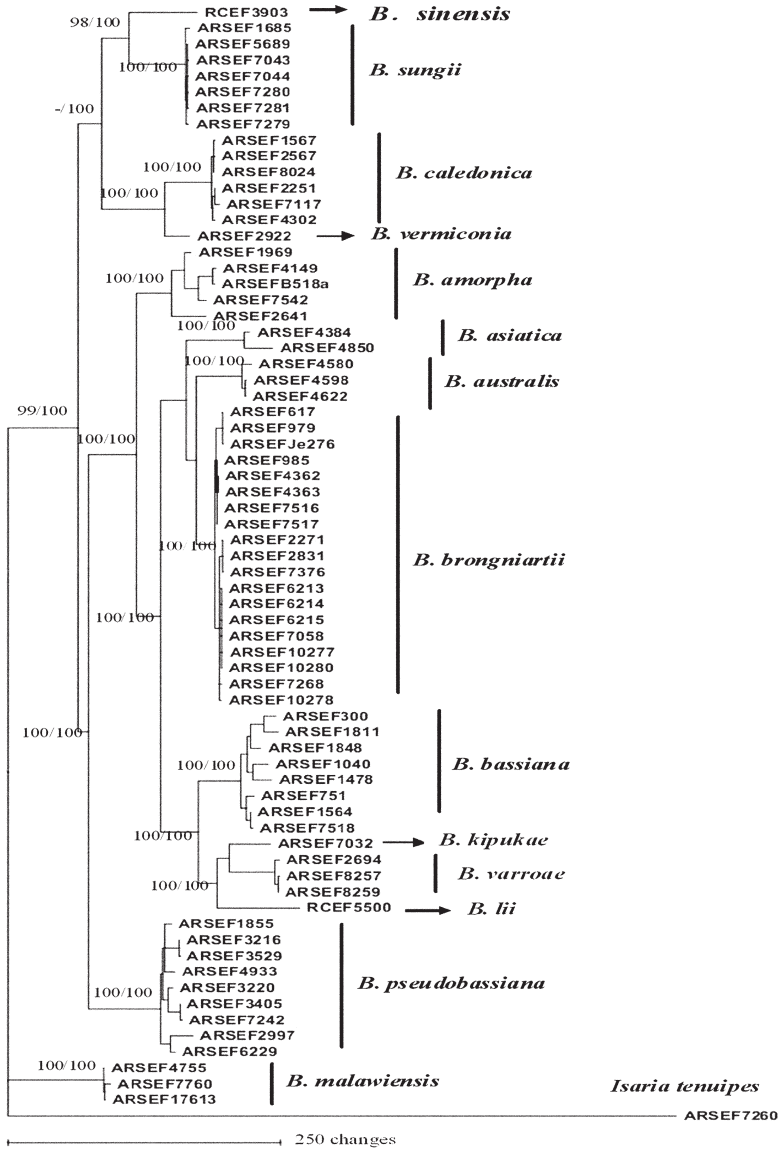
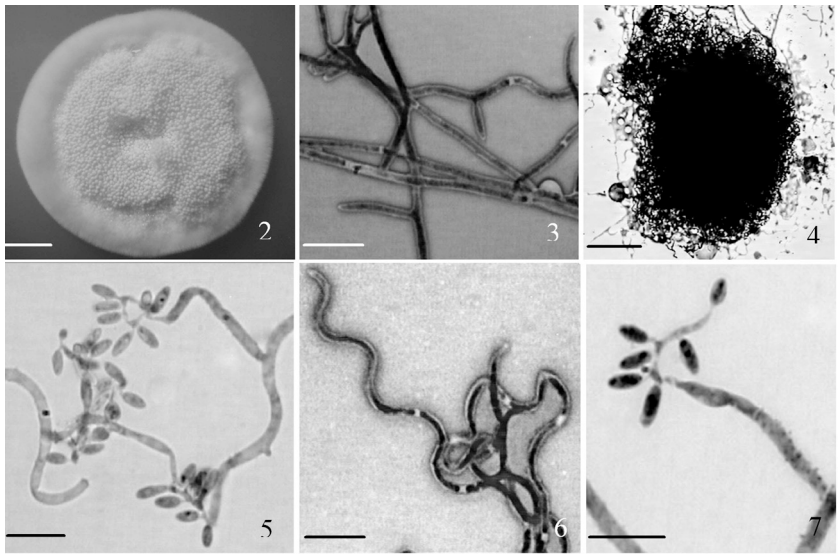


Fig. 1. A single tree from the maximum parsimony analysis showing phylogenetic relationships among species of *Beauveria* inferred from a combined dataset of TEF, RPB1, and RPB2 data. Bootstrap values ≥70% and posterior probabilities ≥95% are labeled above and below the appropriate branches.



Figs 2–7. *Beauveria sinensis.* 2. Colony on SDAY/4, showing mycelial pellets. 3. Typical hyphae. 4. Mycelial pellets. 5. Conidiophores, conidiogenous cells, and conidia. 6. Curved hyphae. 7. Phialide and conidia. Scale bars: 2 = 50 mm; 3, 5, 6, 7 = 10 µm; 4 = 30 µm.

informative nucleotide characters. Therefore, the position of *B. sinensis* in the tree from the combined analysis of RPB1, RPB2, and TEF data is reasonable and reliable.

In culture, *B. sinensis* is distinguishable by a pale pink colony and production of mycelial pellets consisting of curved hyphae. Most species of *Beauveria* are white to buff on solid media except *B. malawiensis,* whose colonies become light pink in older portions. Mycelial pellets consisting of curved hyphae are formed by *B. sinensis* isolates but are unknown in any other *Beauveria* species. Subcultured for five generations, the isolates still produced significant amounts of mycelial pellets. The unique stable growth behavior may prove to be of taxonomic value in *Beauveria*, with additional isolates discovered.

In *Beauveria*, there were three species with ellipsoidal conidia (*B. brongniartii*, *B. asiatica*, and *B. sungii*) and four species with elongate ellipsoidal to cylindrical conidia similar to the new species (*B. amorpha*, *B. caledonica*, *B. malawiensis*, and *B. lii*). Table 1 provides a comparative summary of the main characters of *B. sinensis* and the other four species with elongate ellipsoidal to cylindrical conidia. Microscopically, *B. sinensis* is distinguished by conidiogenous cell shape from *B. amorpha*, *B. caledonica*, and *B. malawiensis,* all of which produce whorls of conidiogenous cells with globose to subglobose bases. *Beauveria malawiensis* is further distinguished by its short and thick rachis, *B. amorpha*

Table 1. Morphological comparisons of *Beauveria* spp. with elongated ellipsoidal to cylindrical conidia.

Species	Colony	Conidia (μm)	Conidial shape	Conidiogenous cell
B. amorpha	White to yellow	3.5–5 × 1.5–2	Cylindrical, often flattened on one side or slightly curved	Whorled, globose to subglobose base, basal part 2–4 μm
B. caledonica	White to cream to dull buff	3–5 × 1–1.8	Ellipsoidal to cylindrical	Simple to whorled, swollen base, 2.6–4.2 × 1.6–3.3 μm
B. lii	White to cream	3.1–10.1 × 1.4–3.6	Ellipsoidal to cylindrical, occasionally obovoid	Solitary, occasionally tight clusters of 2–3, base ellipsoidal to cylindrical 4.8–9.0 μm
B. malawiensis	White to pale pink, slightly zonate	3.7–4.5 × 1.3–1.9	Cylindrical	Whorled, base globose to obpyriform, 2.0–6.6 μm
B. sinensis	White to pale pink, curved mycelium in mycelial pellet	3–5 × 1.5–2	Elongate ellipsoidal to cylindrical	Solitary, slightly or no swollen base, 8.2–20 × 1.6–3.0 μm

has a characteristically shaped conidium that is often slightly curved or flattened on one side (Samson & Evans 1982), and *B. lii* produces the largest conidia (Zhang et al. 2013).

In the Bayesian analysis and maximum parsimony of TEF, RPB1, and RPB2 sequence data, *Beauveria sinensis* was the sister species to *B. sungii* group according to Rehner et al. (2011), and was distinct from other *Beauveria* species with cylindrical conidia. Thus, the phylogenetic studies also supported the recognition of *Beauveria sinensis* as a distinct species.

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